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Role of the Monocyte/Macrophage Cell Lineage in Obesity-Related Insulin Resistance

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ROLE OF THE MONOCYTE/MACROPHAGE CELL LINEAGE IN OBESITY-RELATED INSULIN RESISTANCE

A Masters Thesis Presented

By

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Abstract

Background

Obesity is an important risk factor for resistance to insulin-mediated glucose disposal, and is a precursor of type 2 diabetes and other disorders.

Objectives

To identify molecular pathways in adipose tissue and inflammatory cells that may result in obesity-associated insulin resistance, we exploited the fact that not all obese individuals are prone to insulin resistance. Thus the degree of obesity as a variable was removed by studying obese subjects of similar body mass index (BMI) who are insulin-sensitive (IS) versus insulin-resistant (IR).

Methods

Combining gene expression profiling with computational approaches, we determined the global gene expression signatures of omental and subcutaneous adipose tissue samples obtained from 10 obese-IR and 10 obese-IS patients undergoing gastric bypass surgery. In a secondary study, we isolated monocytes from 4 obese-IR, 3 obese-IS, and 4 nonobese-IS adolescent and young adult subjects for purposes of assessing differences in expression of inflammatory genes in monocytes using RT-PCR.

Results

Gene sets related to chemokine activity and chemokine receptor-binding were identified as most highly enriched in the omental tissue from obese-IR compared to obese-IS subjects, independent of BMI. Strikingly, insulin resistance, but not BMI, was associated with increased macrophage infiltration in the omental adipose tissue, as was adipocyte size.

In the adolescent and young adult cohort, expression of two cytokine signaling molecules (IL8, SOCS3) and two downstream products of the JNK pathway (JunB, c-Fos) showed increased expression in the obese-IR subjects compared to the obese-IS and nonobese-IS subjects, suggesting the presence of a proinflammatory phenotype in monocytes in obesity, which is exacerbated in the insulin resistant state.

Conclusions

Our findings demonstrate that inflammation of omental adipose tissue and activation of proinflammatory monocytes is strongly associated with insulin resistance in human obesity. Manipulation of these pathways may result in the prevention of or delay in the onset of obesity-related co-morbidities.

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List of Symbols, Abbreviations or Nomenclature

BMI : body mass index
CCL18: chemokine (C-C motif) ligand 18
CCL2: chemokine (C-C motif) ligand 2
CCL3: chemokine (C-C motif) ligand 3
CCL4: chemokine (C-C motif) ligand 4
CCR2 : chemokine (C-C motif) receptor 2
CX3CR1: chemokine (C-X3-C motif) receptor 1
CRP: c-reactive protein
DAVID : Database for Annotation, Visualization and Integrated Discovery
GEO: Gene Expression Omnibus
HDL : high density lipoprotein
HFD: high fat diet
HOMA2-IR: homeostatic model of assessment of insulin resistance
IKKβ: inhibitor of NF-κB (IκB) kinase β
IL1: interleukin-1
IL6: interleukin-6
IL8 :interleukin 8
IR: insulin resistance
IRS-1: insulin receptor substrate-1
JAK2-STAT3: Janus-activating kinase2-signal transducer and activator of transcription 3
JNK: c-Jun NH_2-terminal kinase
LDL: low-density lipoprotein
LPS: lipopolysaccharide
MACE: Microarray Computational Environment
MCP1: monocyte chemoattractant protein-1
NF-κB: nuclear factor-kappa B
NHANES: National Health and Nutrition Examination Surveys
Nonobese-IS : nonobese insulin sensitive
Obese-IR : obese insulin resistant
Obese-IS : obese insulin sensitive
RT-PCR: real time-polymerase chain reaction
SOCS3: suppressor of cytokine signaling 3
T2DM: type 2 diabetes mellitus
TLR: Toll-like receptor
TNFα: tumor necrosis factor-α
TYR: tyrosine

CHAPTER I

1. Introduction

1.1 Description of the research problem

The prevalence and severity of obesity are increasing dramatically in the United States placing millions of Americans at risk for obesity-related co-morbidities including type 2 diabetes mellitus (T2DM), and its precursor state of insulin resistance (IR). Interestingly, one-third of obese individuals do not manifest obesity-related co-morbidities, highlighting the importance of identifying specific genetic and biological factors that place certain obese individuals at the greatest risk for obesity-related complications. Adipose inflammation, systemic cytokines and inflammatory cells (such as macrophages) are known to contribute to insulin resistance. In depth exploration of these factors in obese humans with varying degrees of insulin resistance may provide further insights into the pathophysiology of insulin resistance, detect individuals at the greatest risk for developing obesity-related co-morbidities, and identify therapeutic targets to prevent these complications.

1.2 Overview of thesis

Our first objective was to identify genes potentially involved in mediating obesity-related insulin resistance. To this end, we determined the gene expression profiles of subcutaneous and visceral adipose tissue from obese insulin-resistant (obese-IR) and obese insulin-sensitive (obese-IS) adults that had similar body

mass index (BMI) levels. The results from this study identified inflammation and adipose tissue macrophage infiltration as histologic findings that are strongly associated with insulin resistance. This information guided our next objective, namely to assess the role of monocytes, the macrophage precursors, in insulin resistance. To this end, we isolated monocytes from obese-IR, obese-IS, and nonobese-IS adolescents and young adults for purposes of assessing differences in expression of inflammatory genes in monocytes. Results from this study may provide clues regarding the pathophysiology of insulin resistance in young individuals, suggest biomarkers for identifying at-risk individuals, and indicate therapeutic targets for preventing or delaying obesity-related complications.

CHAPTER II

2. Related work (Part 1)

2.1 Obesity and related co-morbidities

The prevalence and severity of obesity are increasing dramatically in the United States and throughout the world[1]. The frequency of obesity more than doubled in adult Americans and the prevalence of overweight individuals more than tripled in U.S. children between 1980 to 2004[2]. According to data from the National Health and Nutrition Examination Surveys (NHANES), 86% of U.S. adults and 30% of U.S. children will be overweight by 2030 if current trends persist[3]. This obesity epidemic places millions of Americans at risk for obesity-related co-morbidities including type 2 diabetes mellitus (T2DM), hypertension, dyslipidemia and coronary artery disease.

2.2 Relationship between insulin resistance, adipose tissue and inflammation

Obesity induced insulin resistance, a precursor to T2DM, is characterized by a decreased response to insulin signaling. The current mechanisms thought to cause insulin resistance are controversial, and involve either ectopic lipid deposition in nonadipose tissues[4], impairment of insulin signaling by circulating cytokines[5], or a combination of the two mechanisms. In both scenarios, adipose tissue plays a major role, either by its inability to expand in order to meet energy storage demands, or its contribution to the elevation of systemic inflammatory

cytokines. Numerous studies have shown that an increase in circulating inflammatory markers in obese subjects, including tumor necrosis factor-α (TNFα), interleukin-6 (IL6) and c-reactive protein (CRP), is associated with the development of diabetes[6,7]. Intensive lifestyle interventions that result in modest weight loss can improve the inflammatory state, reduce markers of inflammation, and delay or prevent the onset of diabetes as evidenced by multiple studies including the Diabetes Prevention Program[8-11].

2.3 Adipose tissue macrophages contribute to insulin resistance

Cytokines are thought to cause insulin resistance by directly impairing muscle insulin signaling and by increasing lipolysis resulting in excess circulating fatty acids and enhanced ectopic lipid deposition in insulin responsive organs[12]. The source(s) of the inflammatory cytokines that are elevated in obesity and obese-IR have yet to be determined. However, several inflammatory cytokines are secreted from adipose tissue, which is comprised of multiple cell types including adipocytes and macrophages. Adipose tissue in obese individuals is characterized by macrophage infiltration, which is thought to be the predominant source of the proinflammatory factors[12]. The ability of both adipocytes and adipose tissue macrophages to secrete circulating cytokines that contribute to insulin resistance highlights the importance of the role adipose tissue plays in glucose homeostasis and whole body metabolism.

2.4 Gene expression data from adipose tissue provide insights into insulin resistance

Despite increasing awareness of the role inflamed adipose tissue plays in obesity related co-morbidities (i.e. insulin resistance, dyslipidemia, hypertension), there is limited understanding of the molecular signals that initiate the inflammatory process. To address this question, most gene expression profiling studies have examined differences in adipose tissue gene expression between obese and lean subjects and found that obesity is associated with an increase in the expression of proinflammatory genes[13,14]. To further complicate matters, adipose tissue is subdivided into subcutaneous and visceral depots, each of which has unique physiologic properties and distinct roles in pathophysiology[5]. Expansion of the visceral adipose depot correlates with an increased risk of cardiovascular disease and diabetes, which may be a function of the close proximity of this adipose depot to the liver via the portal circulation[5]. Increased expression of genes associated with immune and defense responses, particularly monocyte chemoattractant protein 1 (MCP also known as CCL2) was observed in the subcutaneous tissue of obese subjects when compared to lean subjects[13]. A separate study reported increased expression of proinflammatory and adipogenic genes and decreased expression of lipogenic and insulin signaling genes in non-diabetic obese women compared to lean women[14]. However, in both studies, limited information was provided regarding the insulin sensitivity of the participants, so the obese group may have been heterogeneous with respect to

obesity related co-morbidities. Therefore, these studies cannot reveal genes responsible for the metabolically compromised status of some obese individuals compared to other obese individuals. Maclaren, et al showed that changes in expression of genes encoding proteins in the insulin signaling pathway were greater in omental than in subcutaneous tissue, and were related to insulin resistance rather than obesity[15]. However, it is difficult to know if this is a cause of insulin resistance or merely a response to the increased levels of circulating insulin in the insulin resistant population.

2.5 Obese insulin-sensitive phenotype

While obesity places many individuals at risk for multiple co-morbidities, one-third of obese individuals do not manifest obesity-related comorbidities[16,17], highlighting the importance of identifying specific genetic and biological factors that place certain obese individuals at the greatest risk for obesity-related complications. Insulin-sensitive obese patients typically display an early onset of obesity, a fasting insulin level within the normal range, a normal distribution of excess fat, normal triglycerides and increased high density lipoprotein (HDL)-cholesterol when compared to their insulin-resistant obese peers[16]. To identify genes potentially involved in mediating obesity-related insulin resistance, we determined the gene expression profiles of subcutaneous and visceral adipose tissue from obese-IR and obese-IS adults who had similar BMI levels and who were undergoing gastric bypass surgery. Our hypothesis is that candidate genes

identified by differential expression in this screen would be directly related to insulin resistance and not simply a feature of the obese state.

CHAPTER III

3. Details of Research (Part 1)

3.1 Roux-en-Y gastric bypass population

Adult patients undergoing a laparoscopic Roux-en-Y gastric bypass surgery between October 2005 and May 2009 were recruited for this study at the University of Massachusetts Memorial Medical Center. This population of patients demonstrates a wide spectrum of insulin resistance as reported in previous publications[18]. All patients were qualified for gastric bypass surgery under published NIH consensus guidelines for the treatment of morbidly obese patients[19]. Our cohort was restricted to patients with BMI levels between 39 and 55, so the average BMI of the obese-IR patients was similar to the average BMI of the obese-IS patients. Demographic data including age, gender, height, weight, and calculated BMI were measured in the Weight Center. Fasting glucose and insulin levels were recorded at the time of gastric bypass surgery. A lipid profile (triglycerides, total cholesterol, low-density lipoprotein (LDL) cholesterol and HDL cholesterol) was obtained prior to surgery. Patients with diabetes mellitus were excluded from this study because treatment of this metabolic disorder has been shown to alter the gene expression profile[20]. Patients were defined as diabetic if they either used oral hypoglycemic medications or insulin. Adipose tissue samples were taken from lower abdominal wall (subcutaneous) and greater omentum (visceral). All subjects provided written informed consent before taking part in the study. The study was

approved by the University of Massachusetts Medical School Institutional Review Board.

3.2 Technical details

Insulin sensitivity

After an overnight fast, pre-operative serum levels of insulin and glucose were determined. A solid phase chemiluminescent immunoassay was used to determine serum insulin levels (IMMULITE 2000 Insulin), whereas levels of serum glucose were determined by an oxygen rate method employing a Beckman Oxygen electrode (SYNCHRON LX Systems). The homeostatic model of assessment (HOMA2-IR) was used to estimate insulin resistance (http://www.dtu.ox.ac.uk) by using fasting glucose (mg/dL) and fasting insulin levels (mIU/mL). A cut point of HOMA2-IR ≥ 2.3 was used to characterize subjects as being insulin resistant as validated by previous studies in this patient population[18].

Microarray Study

Paired samples of omental and subcutaneous adipose were obtained from fasting patients during gastric bypass surgery. Whole adipose tissue was dissected to remove fibrotic tissue and obvious vasculature, and frozen in 0.5 cm^3 sections in liquid nitrogen. Total RNA was isolated from frozen human tissues by homogenizing the tissue in TRIzol (Invitrogen, Carlsbad, CA). RNA

quality was assessed using a denaturing agarose gel. Total RNA was purified using the RNeasy Mini-Kit (Qiagen, Valencia, CA). Ten micrograms of purified total RNA was prepared according to Affymetrix protocols. Prepared cRNA was hybridized to Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays.

GeneChip Expression Array Analysis was performed as previously published[21] filtering for p-value <0.05 and fold change ≥ 2. This method was used to rank the genes expressed differentially between IS and IR patients in both the subcutaneous and visceral adipose depots. The gene lists that were obtained using the filtering parameters mentioned above were entered into the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics database (http://david.abcc.ncifcrf.gov/home.jsp)[22,23] to determine which molecular functions were most represented in the top-scoring genes using information obtained from the Gene Ontology Consortium (http://www.geneontology.org). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE20950.

Real-time PCR

RNA isolated from the visceral adipose tissue from the twenty patients was analyzed to confirm the microarray analysis findings. cDNA was synthesized using iScript cDNA Synthesis Kit (BioRad, Hercules, CA). Quantitative real-time

PCR for specific genes was performed using SybrGreen normalized to beta 2 microglobulin. Primers were designed using PrimerBank which is a public resource for PCR primers using a primer design algorithm that has been tested by real-time PCR experiments for PCR specificity and efficiency (pga.mgh.harvard.edu/primerbank)[24]. Melting curve data was collected to visualize the presence of primer dimers. We used the following primers for RT-PCR: CCL2-sense, 5'-CCC CAG TCA CCT GCT GTT AT-3'; CCL2-antisense, 5'-TGG AAT CCT GAA CCC ACT TC-3'; IL8-sense, 5'-ACT GAG AGT GAT TGA GAG TGG AC-3'; IL8-antisense, 5'-AAC CCT CTG CAC CCA GTT TTC-3'; CCL3-sense, 5'-AGT TCT CTG CAT CAC TTG CTG-3'; CCL3-antisense, 5'-CGG CTT CGC TTG GTT AGG AA-3'; CCL4-sense, 5'-CTG TGC TGA TCC CAG TGA ATC-3'; CCL4-antisense, 5'TCA GTT CAG TTC CAG GTC ATA CA-3'; CCL18-sense, 5'-GGG GGC TGG TTT CAG AAT A-3', CCL18-antisense, 5'-CTC CTT GTC CTC GTC TGC AC-3'; Beta2M-sense, 5'-GGC TAT CCA GCG TAC TCC AAA-3'; Beta2M-antisense, 5'-CGG CAG GCA TAC TCA TCT TTT T-3'. Expression of specific mRNAs was quantified in duplicate samples on an iCycler IQ Real-Time PCR detection system (Bio-Rad Laboratories) using the $\Delta\Delta C_T$ method.

Adipose tissue histology

Tissues were fixed in 10% buffered formalin and embedded in paraffin from which multiple 5-mm sections were obtained. Images were acquired using an

Axioskop2 Plus (Zeiss, Göttingen, Germany) coupled to a Spot RTKe (Diagnostic Instruments, Inc) and were analyzed using SPOT 4.0.2 software. For each subject, adipocyte cell diameter was calculated from the perimeter measurement (Adobe Photoshop CS4 Extended) and averaged using 100 cells. Immunohistochemical detection of CD68 (Santa Cruz Biotechnologies, Heidelberg, Germany) diluted 1:50 was performed with the avidin-biotin peroxidase method. Adipocytes and CD68+ cells were counted at 10x magnification in ten different randomly chosen areas for omental adipose tissue, and five randomly chosen areas for subcutaneous adipose tissue (due to the limited size of this tissue sample).

Statistical analysis

Baseline differences between insulin sensitive and insulin resistant subjects were evaluated using the Student's *t* test for continuous variables and Fisher's exact test for categorical variables. Variables with a skewed distribution (total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, fasting glucose, fasting insulin, HOMA2) were log-transformed before analysis. For genes analyzed by real time-PCR (RT-PCR), significant differences between insulin sensitive and insulin resistant groups were determined by Student's *t* test. The results were considered significant at $P \leq 0.05$. For Gene Ontology Molecular Function gene sets analyzed by DAVID, significant differences between insulin sensitive and insulin resistant groups were determined using the Bonferroni

correction to address the problem of multiple comparisons. The results were considered significant at Bonferroni $P \leq 0.05$. The relationships between subject clinical data (HOMA2-IR or BMI) and characteristics of adipose histology (macrophage infiltration or adipocyte diameter) were determined by regression analysis.

CHAPTER IV

4. Experimental Evaluation (Part 1)

4.1 Clinical and metabolic characteristics of the study population

Adipose tissue samples from 20 subjects were used in this study. Fourteen of the twenty subjects were female and the average age of the study sample was 42 years (Table 4.1). Using HOMA2-IR analysis, we divided the subjects into two groups: insulin sensitive (n=10) and insulin resistant (n=10). Insulin resistant subjects showed significantly elevated HOMA2-IR as compared to insulin sensitive subjects (see Methods). In addition, both fasting insulin ($p < 0.001$) and fasting glucose ($p = 0.02$) levels were higher in insulin resistant subjects. The obese-IR group has elevated triglyceride levels compared with the obese-IS group, which is not unexpected. While the 2 groups had similar total cholesterol and LDL cholesterol levels, two of the obese-IR patients with low total cholesterol and LDL cholesterol levels were on lipid lowering medications which are known to lower LDL values (Figure 4.1). This may account for the similarity in cholesterol levels between the two groups.

4.2 Global gene expression profiling of adipose tissue identifies active chemokine signaling in obese-insulin resistant patients

In order to identify genes related to insulin resistance rather than obesity *per se*, we first utilized cDNA microarrays to determine the gene expression profiles of paired subcutaneous and omental samples from the 20 obese subjects (one

insulin resistant subject did not have a subcutaneous sample available for microarray analysis). Using GeneChip Expression Array Analysis and filtering parameters p-value < 0.05 and fold change ≥ 2, we identified 37 genes differentially expressed in omental tissue of insulin resistant subjects as compared to insulin sensitive subjects, and three genes differentially expressed in subcutaneous tissue between the two subject groups (Table 4.2, Figure 4.2).

To elucidate the molecular functions differentially regulated in the omental adipose tissue of our subjects, we entered the gene list into the DAVID bioinformatics database and identified 2 Gene Ontology Molecular Function gene sets as being differentially expressed between the insulin resistant and insulin sensitive subjects (Table 4.3). The gene sets representing chemokine activity and chemokine receptor binding were upregulated in omental tissue of insulin resistant subjects and reached statistical significance using the Bonferroni correction to address the problem of multiple comparisons. Both gene sets included the genes chemokine (C-C motif) ligand 18 (CCL18), chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 3 (CCL3), chemokine (C-C motif) ligand 4 (CCL4) and interleukin 8 (IL8). These five genes were studied with quantitative RT-PCR analysis which revealed that the direction and magnitude of the differential expression in omental tissue was consistent with the data provided by microarray analysis, thereby confirming the computational approach (Fig 4.3). These trends were upheld when the data were re-analyzed

using just the female patients, confirming that the observed differences were not due to gender bias (Figure 4.4). While the expression of these five genes, as assessed by signal intensity on the Affymetrix gene chip, was much higher in the subcutaneous tissue, there was no difference in expression between the obese-IR and obese-IS groups (Table 4.4).

4.3 Adipose tissue macrophage infiltration and adipocyte size

To determine whether the differences in chemokine expression with insulin resistance were related to changes in adipose tissue composition, we performed an immunohistochemical analysis of omental adipose tissue samples from eleven patients which comprised a subset of the original cohort of twenty patients. We observed strikingly larger adipocyte size and increased macrophage infiltration associated with insulin resistance. (Figures 4.5 4.6) There was a significant correlation between HOMA2-IR and macrophage infiltration (Fig 4.7) on the other hand, analysis of CD68 staining with BMI in the morbidly obese range showed no association (Fig 4.9) Adipocyte diameter was inversely correlated with HOMA2-IR (Figure 4.8) but not with BMI (Figure 4.10) in our obese subjects.

Immunohistochemical analysis was performed on the subcutaneous tissue of seven patients for comparison (the subcutaneous tissue from the other four

patients was poor quality). On average, the adipocytes were smaller than those observed in the omental tissue, there were fewer macrophages, and there was not correlation between HOMA2-IR and adipocyte size or macrophage infiltration (Figs 4.11 – 4.14).

4.4 Conclusions

In this study we aimed to identify molecular pathways in adipose tissue related directly to insulin resistance in obese adult men and women. By combining expression profiling with computational approaches, we found that elevated chemokine and chemokine receptor activity distinguishes the omental adipose tissue of insulin resistant obese patients from omental adipose of insulin sensitive obese individuals. In addition, our histological analysis determined that increased adipocyte size and increased macrophage infiltration of the adipose tissue is associated with insulin resistance and not simply BMI in morbidly obese human subjects.

Multiple chemokines play a role in macrophage recruitment to adipose tissue

Both human and animal studies demonstrate that hypertrophied adipocytes secrete CCL2, which is a chemoattractant that increases macrophage infiltration[25,26,27]. As such, CCL2 is thought to be the primary chemokine secreted from adipose tissue in the obese state, and responsible for adipose inflammation leading to subsequent metabolic alterations[13]. However, CCL2-

deficient mice do not have diminished macrophage infiltration or improved glucose metabolism compared to control mice[28], suggesting that other chemokines may play critical a role in the adipose inflammation associated with obesity. The increased expression of five chemokines in the visceral adipose tissue of our insulin resistant cohort supports this theory, which has been previously demonstrated in studies of obese subjects with lean controls[29]. Of note, CCL2 did not have the highest fold change of the chemokines we identified as being upregulated in insulin resistance. Interleukin 8/CXCL8, another chemokine identified in our insulin resistant sample, is differentially secreted by visceral adipose tissue and implicated in coronary heart disease[30] suggesting importance in obesity related sequelae.

Adipocyte hypertrophy is associated with insulin resistance

Enlarged adipocyte size is associated with insulin resistance[31], increased adipokine production and secretion[32,33]. In our study population, insulin resistance was associated with an increase in adipocyte size. Chemokines, secreted by hypertrophied adipocytes, attract macrophages that can be seen dispersed throughout the tissue, or clustered around adipocytes in “crown like structures” and secrete cytokines that contribute to the low-grade inflammation seen in obesity. Omental fat appears to have more macrophage infiltration than subcutaneous fat, a finding that is exaggerated by central obesity[34]. Our data

support the hypothesis that the relationship between insulin resistance, adipocyte size and macrophage infiltration is not a function of overall weight, but perhaps related to one or more unknown factors including adipose distribution or visceral adiposity.

Study strengths and limitations

The strengths of our study consist of the inclusion of obese subjects with a range of insulin sensitivity and the use of microarray data, which allows for the unbiased analysis of the expression of multiple genes in different pathways. By restricting the BMI of the obese-IS and obese-IR groups we reduced the influence of obesity itself as a variable in examining the association between insulin resistance with altered gene expression. Yet, we recruited a morbidly obese population which may raise potential concerns with generalizability of findings to a population with a moderate or mild degree of obesity. One may argue that the measurement of BMI may not accurately reflect the degree of body fat content and may be strengthened by more objective data on body fat composition or waist circumference. However, recent evidence[35] suggests that even waist circumference is not a useful measurement in persons with severe obesity (BMI $\geq$ 40). Thus this issue remains open for further analysis in future studies. Due to our limited group of surgical patients we included both female and male subjects in both the insulin resistant and insulin sensitive cohorts. Yet, the enrichment of genes encoding chemokines in the omental adipose tissue of

insulin resistant patients was observed even when we limited our analysis to the female patients (Figure 4.3), making it unlikely that our observed differences were due to gender bias.

Our data are consistent with a growing body of evidence suggesting that adipose inflammation plays a role in mediating the insulin resistance itself, as it is manifest in obesity. We showed that omental adipose chemokine expression and macrophage infiltration is associated with insulin resistance, independent of obesity in a morbidly obese population. This study raises the important question: why do some obese patients display such inflammation in their omental adipose tissue, while other equally obese patients do not? Pharmaceutical agents aimed at interrupting the chemokine pathway may be able to diminish the metabolic sequelae associated with obesity.

Tables

Table 4.1. Clinical and Biochemical Characteristics of the Study Participants*

	Insulin Sensitive HOMA < 2.3 (N= 10)	Insulin Resistant HOMA >= 2.3 (N= 10)	P Value
Age - yr			0.2
Mean	39 ± 6	44 ± 9	
Range	31-54	33-57	
Female sex - no. (%)	8 (80)	6 (60)	0.4
BMI§			0.7
Mean	48 ± 3	49 ± 7	
Range	46-55	39-60	
Total cholesterol (mg/dL)**			0.9
Mean	176 ± 27	179 ± 50	
Range	115-211	111-276	
LDL cholesterol (mg/dL)**			0.4
Mean	109 ± 15	101 ± 45	
Range	76-127	45-190	
HDL cholesterol (mg/dL)**			
Mean	44 ± 5	42 ± 9	0.4
Range	38-52	30-56	
Triglycerides (mg/dL)**			0.4
Mean	129 ± 33	166 ± 74	
Range	94-188	74-274	
Lipid lowering therapy - no. (%)	1 (10)	3 (30)	0.3
Fasting glucose (mg/dL)			0.02
Mean	88 ± 9	101 ± 12	
Range	75-100	82-127	
Fasting insulin (mIU/mL)			<0.001
Mean	9 ± 4	24 ± 7	
Range	4-14	16-36	
HOMA2			<0.001
Mean	1.3 ± 0.5	3.6 ± 1	
Range	0.6-2.1	2.4-5.3	

* Plus-minus values are mean ± SD.

§ The body-mass-index (BMI) is the weight in kilograms divided by the square of the height in meters.

** Data was not available for three patients (1 IS, 2 IR)

Table 4.2. Individual genes differentially expressed in adipose tissue from insulin resistant compared to insulin sensitive obese patients.

Probe ID	Gene name	Symbol	FC	*P* value
OMENTAL DEPOT				
203936_s_at	matrix metallopeptidase 9	MMP9	2.9	0.001
206932_at	cholesterol 25-hydroxylase	CH25H	2.8	0.014
216598_s_at	chemokine (C-C motif) ligand 2	CCL2	2.7	0.013
202859_x_at	interleukin 8	IL8	2.7	0.023
209395_at	chitinase 3-like 1 (cartilage glycoprotein-39)	CHI3L1	2.7	0.007
205114_s_at	chemokine (C-C motif) ligand 3	CCL3	2.6	0.029
212657_s_at	interleukin 1 receptor antagonist	IL1RN	2.4	0.005
209875_s_at	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1	2.2	0.044
230040_at	ADAM metallopeptidase with thrombospondin type 1 motif, 18	ADAMTS18	2.2	0.044
205681_at	BCL2-related protein A1	BCL2A1	2.1	0.004
213975_s_at	lysozyme (renal amyloidosis)	LYZ	2.1	0.016
202499_s_at	solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	2.1	0.021
209959_at	nuclear receptor subfamily 4, group A, member 3	NR4A3	2.1	0.024
204103_at	chemokine (C-C motif) ligand 4	CCL4	2.0	0.044
224009_x_at	dehydrogenase/reductase (SDR family) member 9	DHRS9	2.0	0.005
32128_at	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	CCL18	2.0	0.043
229160_at	melanoma associated antigen (mutated) 1-like 1	MUM1L1	-2.0	0.019
242662_at	Proprotein convertase subtilisin/kexin type 6	PCSK6	-2.0	0.044
232164_s_at	epiplakin 1	EPPK1	-2.0	0.024
204364_s_at	receptor accessory protein 1	REEP1	-2.0	0.042
229796_at	SIX homeobox 4	SIX4	-2.0	0.002
230867_at	collagen type VI alpha 6	COL6A6	-2.0	0.015
1555230_a_at	Kv channel interacting protein 2	KCNIP2	-2.0	0.015
219895_at	family with sequence similarity 70, member A	FAM70A	-2.0	0.034
224480_s_at	1-acylglycerol-3-phosphate O-acyltransferase 9	AGPAT9	-2.1	0.000
205769_at	solute carrier family 27 (fatty acid transporter), member 2	SLC27A2	-2.1	0.013
205960_at	pyruvate dehydrogenase kinase, isozyme 4	PDK4	-2.2	0.025
205433_at	butyrylcholinesterase	BCHE	-2.2	0.046
234103_at	Potassium channel, subfamily T, member 2	KCNT2	-2.2	0.006
204469_at	protein tyrosine phosphatase, receptor-type, Z polypeptide 1	PTPRZ1	-2.3	0.001
206423_at	angiopoietin-like 7	ANGPTL7	-2.3	0.003
209309_at	alpha-2-glycoprotein 1, zinc-binding	AZGP1	-2.3	0.000
214584_x_at	acetyl-Coenzyme A carboxylase beta	ACACB	-2.4	0.001
233314_at	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	PTEN	-2.4	0.019
209470_s_at	glycoprotein M6A	GPM6A	-2.4	0.002
222853_at	fibronectin leucine rich transmembrane protein 3	FLRT3	-2.5	0.019
204560_at	FK506 binding protein 5	FKBP5	-2.8	0.010
SUBCUTANEOUS DEPOT				
214587_at	collagen, type VIII, alpha 1	COL8A1	-2.0	0.031
230867_at	collagen type VI alpha 6	COL6A6	-2.1	0.024
1554062_at	Xg blood group	XG	-2.2	0.045

* Analysis was performed from Affymetrix GeneChip data using Microarray Computational Enviroment 2.0 developed by the Diabetes and Endocrinology Research Center at the University of Massachusetts Medical School. Shown are all named genes significantly upupregulated 2 fold or more. Fold change indicates the difference in expression in insulin resistant as compared to insulin sensitive obese subjects. The P values were not adjusted for multiple testing. $P < 0.05$ was considered statistically significant. Genes with an Absent call were excluded.

Table 4.3. Gene Ontology Molecular Function Terms Enriched in Omental Adipose Tissue from Insulin Resistant Compared to Insulin Sensitive Obese Patients.*

Term	Gene Count	%	*P* value	Bonferroni
chemokine activity	5	13.5	5.3E-06	2.2E-04
chemokine receptor binding	5	13.5	5.8E-06	2.4E-04

* Analysis was performed from Affymetrix GeneChip data using Database for Annotation Visualization and Integrated Discovery 6.7 available on the National Institute of Allergy and Infectious Diseases, NIH website. Shown are all molecular function gene terms with a significant enrichment. The P value was adjusted for multiple testing. Bonferroni $P < 0.05$ was considered statistically significant.

Table 4.4. Affymetrix gene chip signal intensities for five inflammatory genes from omental and subcutaneous tissues.

	Omental				Subcutaneous			
Symbol	IR signal	IS signal	FC	P	IR signal	IS signal	FC	P
CCL2	579	214	2.7	0.013	1497	1128	1.3	NS
IL8	38	14	2.7	0.023	227	247	1.1	NS
CCL3	161	63	2.6	0.029	198	252	1.3	NS
CCL4	276	140	2	0.044	329	373	1.1	NS
CCL18	387	196	2.0	0.043	470	548	1.2	NS

Figure Legend

Figure 4.1 Serum levels of total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides in obese-IR (red dots) and obese-IS (blue squares) subjects. Subjects receiving pharmacologic treatment with a lipid lowering agents are represented by a dark center within their symbol

Figure

4.1

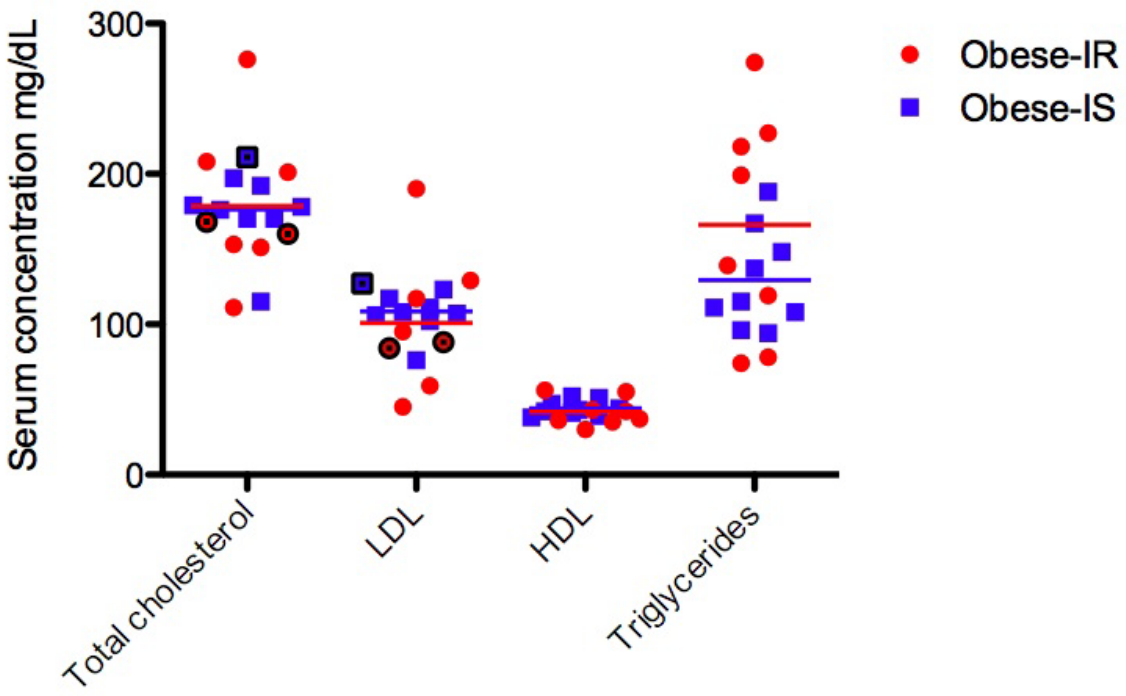

Serum concentration mg/dL
300
200
100
0
Total cholesterol
LDL
HDL
Triglycerides
Obese-IR
Obese-IS

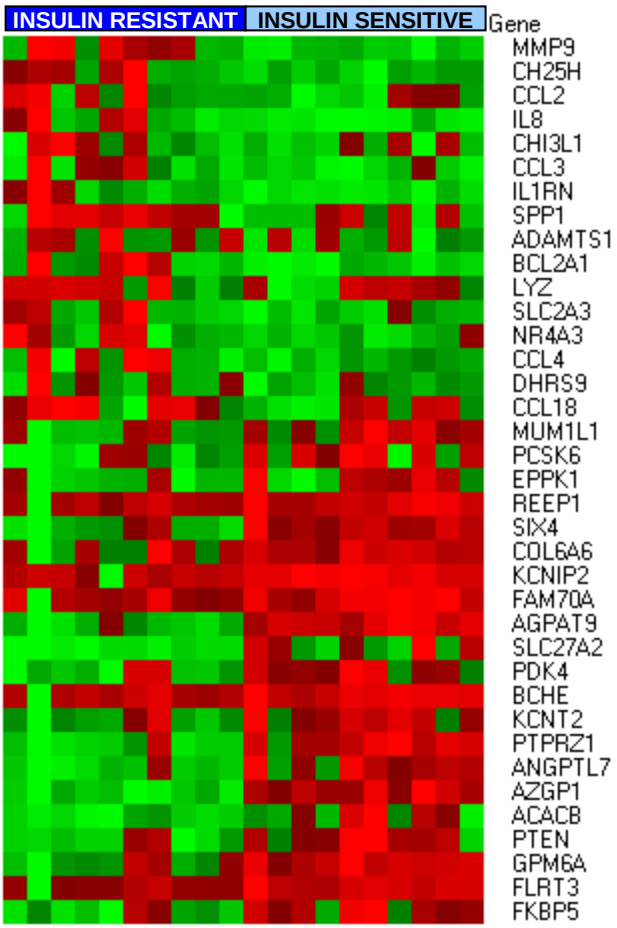
Figure Legend

Figure 4.2. Expression of inflammation-related genes in omental adipose tissue from insulin resistant and insulin sensitive obese human subjects using microarray data. Heatmap representing normalized expression of genes identified by Microarray Computational Environment (MACE) as being significantly increased in omental adipose tissue from insulin resistant subjects and insulin sensitive human subjects. The genes are listed on the right. Expression levels above the mean for the gene are shown in red and expression levels below the mean for the gene are shown in green.

Figure 4.3. Expression of inflammation-related genes in omental adipose tissue from insulin resistant and insulin sensitive obese human subjects using RT-PCR data. Fold change in mRNA level of genes in omental adipose tissue of obese, insulin-resistant subjects (n=10) relative to obese, insulin-sensitive subjects (n=10) based on microarray data (white bars) and quantitative real-time analysis (black bars) performed by RT-PCR. ** $P < 0.01$; * $P < 0.05$.

Figures

4.2



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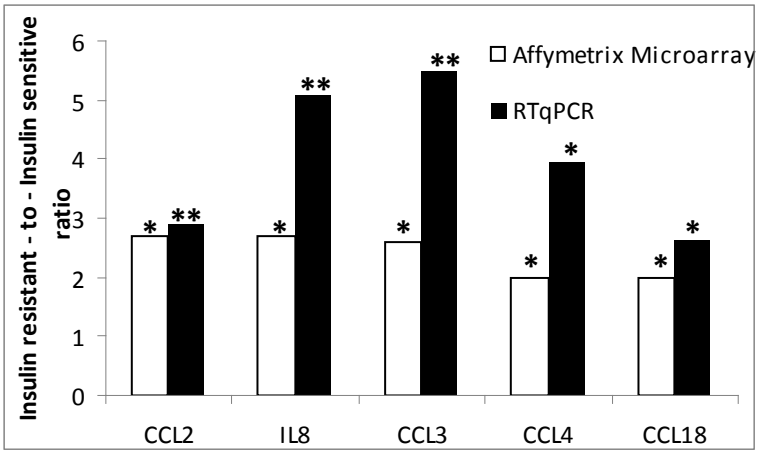


Figure Legend

Figure 4.4. Expression of inflammation-related genes in omental adipose tissue from insulin resistant and insulin sensitive female obese human subjects using microarray and RTqPCR. Fold change in mRNA level of genes in omental adipose tissue of female obese, insulin-resistant subjects (n=6) relative to female obese, insulin-sensitive subjects (n=8) based on microarray data (white bars) and quantitative real-time analysis (black bars) performed by RTqPCR. * $P < 0.05$.

4.4

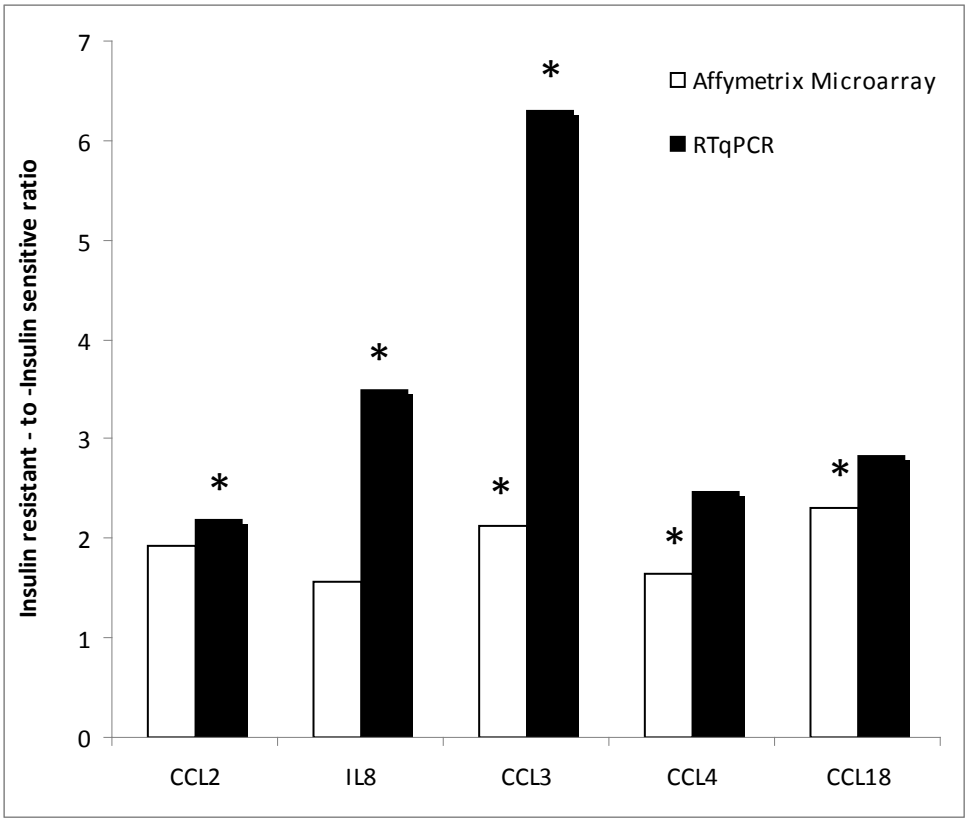

Insulin resistant - to -Insulin sensitive ratio
7
6
5
4
3
2
1
0
Affymetrix Microarray
RTqPCR
*
*
*
*
*
CCL2
IL8
CCL3
CCL4
CCL18

Figure Legend

Relationship between macrophage infiltration, adipocyte diameter and HOMA2-IR in **omental** adipose tissue. Immunohistochemical detection of CD68+ macrophages performed on omental adipose tissue samples obtained from obese human subjects undergoing gastric bypass surgery.

Figure 4.5. CD68 staining of omental adipose tissue from a representative insulin sensitive subject.

Figure 4.6. CD68 staining of omental adipose tissue from a representative insulin resistant subject. Unlike in adipose tissue from insulin sensitive subject (4.4) macrophages are observed throughout the tissue (arrows) and arranged in crown-like structures (arrowhead) in the insulin resistant subject (4.5). Sections taken at 20x magnification.

Quantitative analysis of adipocyte diameter and macrophage infiltration in adipose tissue of insulin resistant and insulin sensitive obese human subjects.

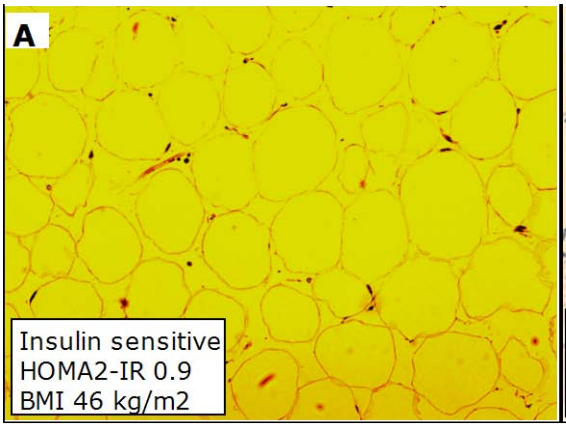
Figure 4.7. Insulin sensitivity, as determined by HOMA2-IR, correlates with CD68+ macrophage infiltration. Data is an average of 10 histological fields using 10X objective.

Figure 4.8. Insulin sensitivity, as determined by HOMA2-IR, correlates with adipocyte diameter. Adipocyte diameter was calculated from the perimeter measurement of 100 cells.

Figure 4.9. Body mass index (BMI) shows no correlation with macrophage infiltration. Data is an average of 10 histological fields using 10X objective.

Figure 4.10. Body mass index (BMI) shows no correlation with adipocyte diameter. Adipocyte diameter was calculated from the perimeter measurement of 100 cells.

4.5



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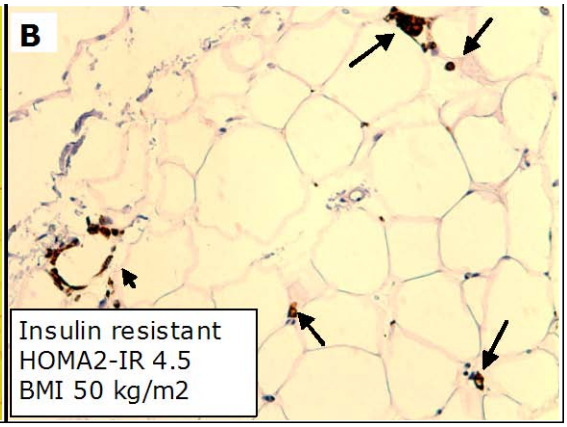

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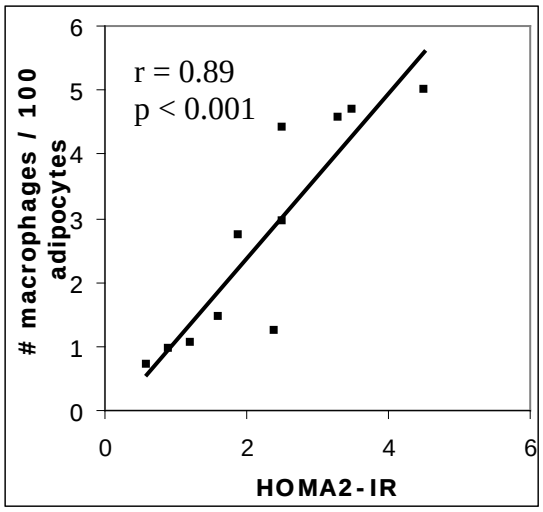

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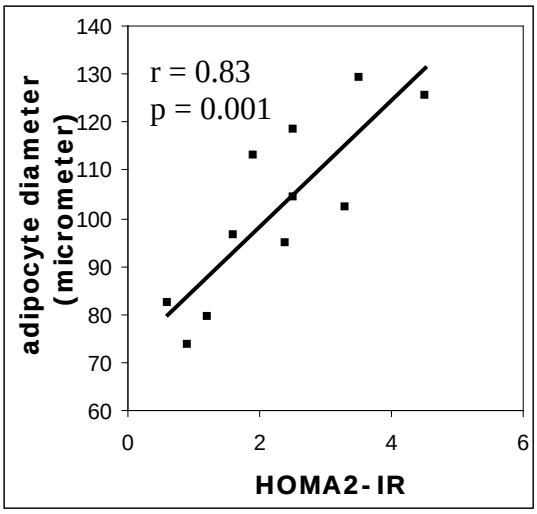

4.9

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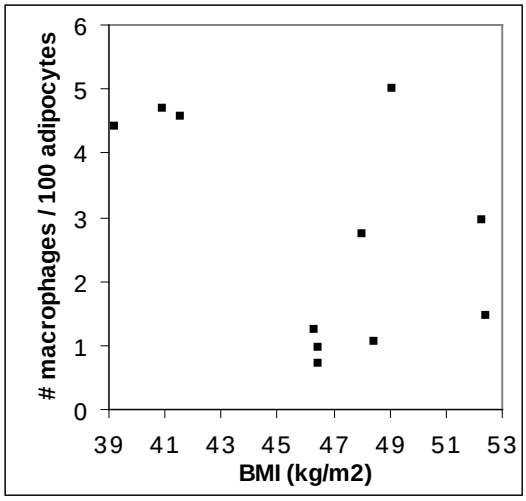
.74



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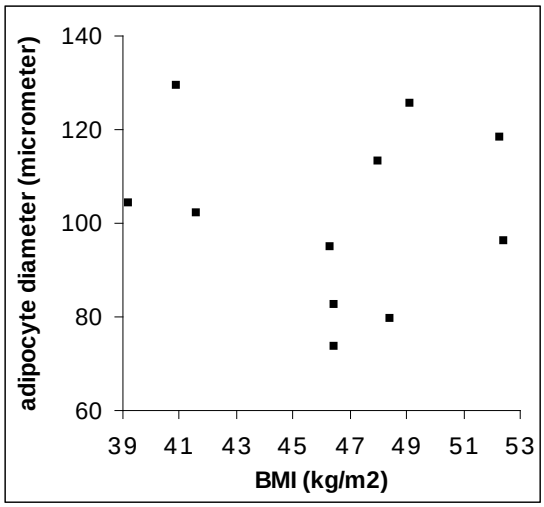


Figure Legend

Relationship between macrophage infiltration, adipocyte diameter and HOMA2-IR in **subcutaneous** adipose tissue. Immunohistochemical detection of CD68+ macrophages performed on subcutaneous adipose tissue samples obtained from obese human subjects undergoing gastric bypass surgery.

Figure 4.11. Insulin sensitivity, as determined by HOMA2-IR, does not correlate with CD68+ macrophage infiltration. Data is an average of 5 histological fields using 10X objective.

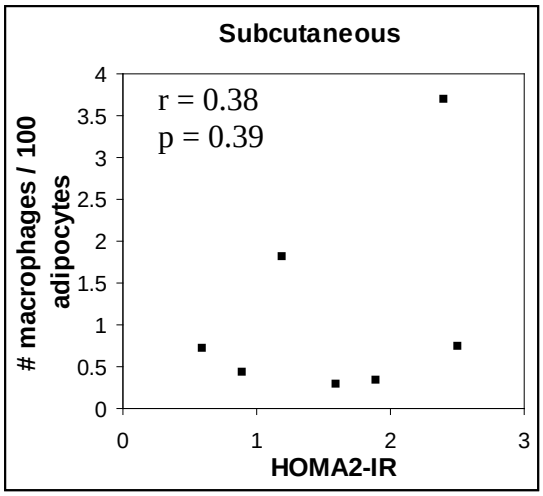
Figure 4.12. Insulin sensitivity, as determined by HOMA2-IR, does not correlate with adipocyte diameter. Adipocyte diameter was calculated from the perimeter measurement of 100 cells.

Figure 4.13. Body mass index (BMI) shows no correlation with macrophage infiltration. Data is an average of 5 histological fields using 10X objective.

Figure 4.14. Body mass index (BMI) shows no correlation with adipocyte diameter. Adipocyte diameter was calculated from the perimeter measurement of 100 cells.

Figures

4.11



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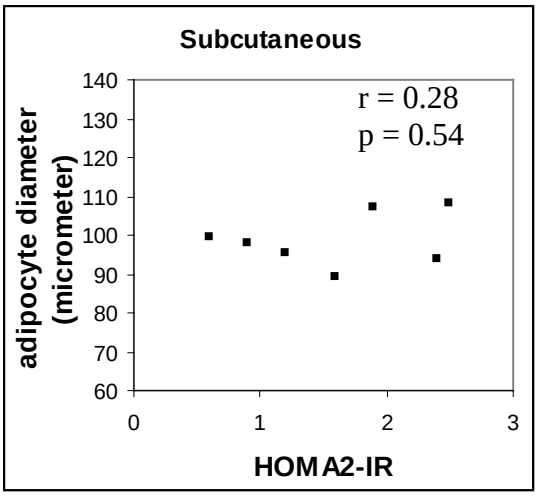

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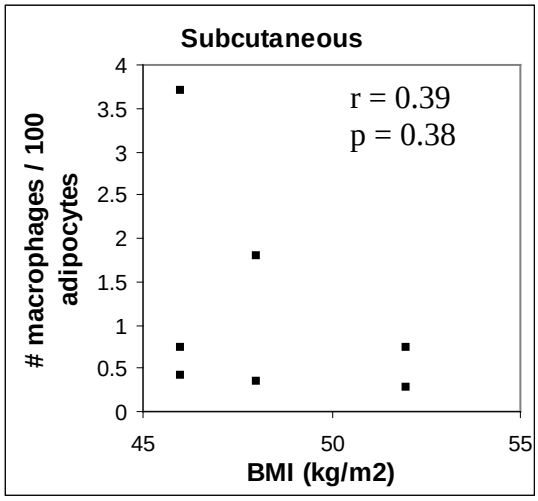

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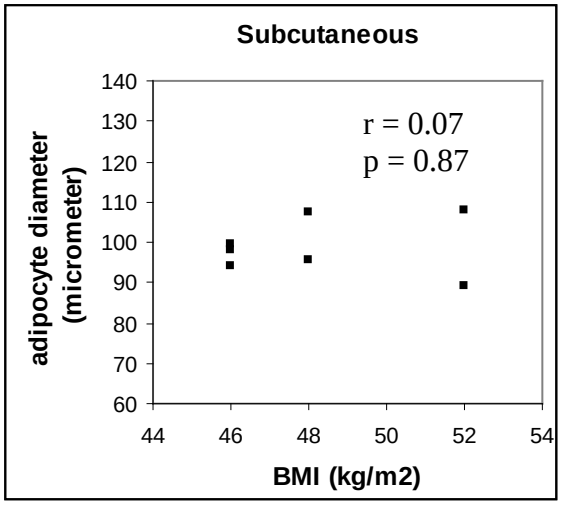


CHAPTER V

5. Related work (Part 2)

5.1 The monocyte/macrophage cell lineage

While the role of the adipose tissue macrophage in the regulation of insulin sensitivity in obese diabetic adults has been described[5], less is known about the molecular response of the peripheral blood monocyte (macrophage precursor) in obesity-induced IR. Monocytes comprise 10% of the leukocytes in human blood, and play an active role in the cellular innate immune system and the inflammatory response[36]. Chronic activation of innate immunity can lead to the undesired development of inflammatory diseases, most notably atherosclerosis. In the setting of obesity associated IR, monocytes can serve as a source of myeloid precursors for the renewal of adipose tissue macrophages[36]. The receptors located on the surface of the monocytes detect a variety of stimuli, including lipids, which increase in response to a high fat diet[36]. Monocytes are easily sampled, and may provide insight that can be used to prevent or delay the inflammation associated with obesity and insulin resistance.

5.2 Monocyte subtypes

Monocytes, as a class, are heterogeneous and differential expression of cell surface markers and chemokine receptors distinguish the 2 major subsets. Proinflammatory $CD14^{+}CD16^{+}$ monocytes are more involved in the inflammatory response than their classical $CD14^{+}CD16^{-}$ counterparts[37]. The $CD14^{+}CD16^{-}$

monocytes comprise 80-90% of monocytes and produce the anti-inflammatory cytokine IL10 in response to lipopolysaccharide (LPS) stimulation and free fatty acids. In contrast, proinflammatory $CD14^{+}CD16^{+}$ monocytes produce TNFα and IL-1 in response to LPS stimulation[36]. The expression of specific chemokine receptors, particularly chemokine (C-C motif) receptor 2 (CCR2) and chemokine (C-X3-C motif) receptor 1 (CX3CR1), also distinguishes the two monocyte subsets[36]. Genetic manipulation of these chemokine receptors in rodent models has shown their importance in glucose metabolism. CCR2 knockout mice have reduced macrophage infiltration into adipose tissue and demonstrate an improvement in glucose homeostasis and insulin sensitivity[38].

5.3 Monocyte activation

Circulating monocytes are easily accessible and their molecular profiles may provide biological and clinical insights into mechanisms of disease. There is a growing literature on gene expression profiling of monocytes in patients with Type 1 diabetes and T2DM. Monocytes from patients with Type 1 diabetes demonstrate increased toll-like receptor expression, upregulation of (NF-κB), and elevated secretion of TNF-α, IL6 and IL-1β[39,40] as compared to non-diabetic subjects. Monocytes from both Type 1 and Type 2 diabetic patients display increased expression of the monocyte chemoattractant protein-1 (MCP-1) gene when compared to non-diabetic controls[41]. Monocytes from subjects with Type 2 diabetes exhibit increased proatherogenic activity compared with those from

matched control subjects[42,43]. Even monocytes from obese metabolically normal individuals are shown to be in an inflammatory activated state when compared to lean individuals[44].

5.4 Inflammatory pathways

The inflammatory cytokines that are elevated in obesity and obese-IR as well as lipotoxic stress from circulating long-chain saturated free fatty acids and lipids activate at least two transcription factor-signaling pathways in adipocytes, hepatocytes and macrophages: the NF-κB pathway which is activated upon degradation of the inhibitor of NF-κB (IκB) kinase β (IKKβ), and the c-Jun NH_2-terminal kinase (JNK) pathway[5,45] (Fig 5.1). Activation of the NF-κB and JNK pathways results in increased expression of NF-κB and AP-1 target gene products including IL6, TNFα, IL1β, MCP-1, and IL8, some of which act as mediators of IR. JNK also exerts its effects through inhibition of tyrosine phosphorylation of insulin receptor substrate-1 which has a negative effect on downstream insulin signaling.

Genetic disruption of these signaling pathways in rodent models has been shown to improve obese-IR in the setting of a high fat diet (HFD), highlighting their importance in this disease process. *JNK1* knockout mice are protected against diet-induced or genetically induced IR[46]. Recently, it was shown that *JNK1* ablation in adipose tissue decreased IL6 production which subsequently lowered

expression of SOCS3, a protein known to mediate hepatic insulin resistance[47]. Additional studies have shown that JNK and NFκB activation in hematopoietically derived cells plays a major role in HFD-induced inflammation and IR with little impact on obesity. Targeted *JNK1* deletion in the hematopoietic compartment protects against HFD-mediated IR by altering obesity-induced inflammation[45], and cell-specific deletion of IKKβ in myeloid cells protects mice from diet-induced IR[48]. A two-week trial of salsalate, a modified form of salicylate which inhibits the IKKβ/NF-κB axis, was recently shown to improve insulin sensitivity and decrease biomarkers of inflammation in a cohort of obese diabetic patients[49]. This supports the possibility of targeting NF-κB as a therapeutic approach in T2DM.

5.5 Childhood obesity

Currently, there are no published studies that have examined the molecular response of monocytes in young obese-IR individuals. One in six overweight adolescents has IR[50], placing this vulnerable population at increased risk for diabetes and heart disease, which are two of the leading causes of morbidity and mortality in the U.S. All children experience a period of physiologic insulin resistance during puberty due to changes in body composition and hormone levels[51], which for some may trigger the onset of pathologic insulin resistance that could progress to type 2 diabetes mellitus. In addition, these adolescents are free from other inflammation-related co-morbidities (arthritis, chronic obstructive pulmonary disease) and are not taking lipid lowering medications,

making them an ideal group to study. To the extent possible, matching on BMI minimizes the impact of obesity as a potential confounding variable. Targeting behavioral modifications and pharmaceutical interventions to treat young adolescents at highest risk will hopefully prevent or delay the development of diabetes and its complications, which will have dramatic implications for the affected individuals as well as their families and society.

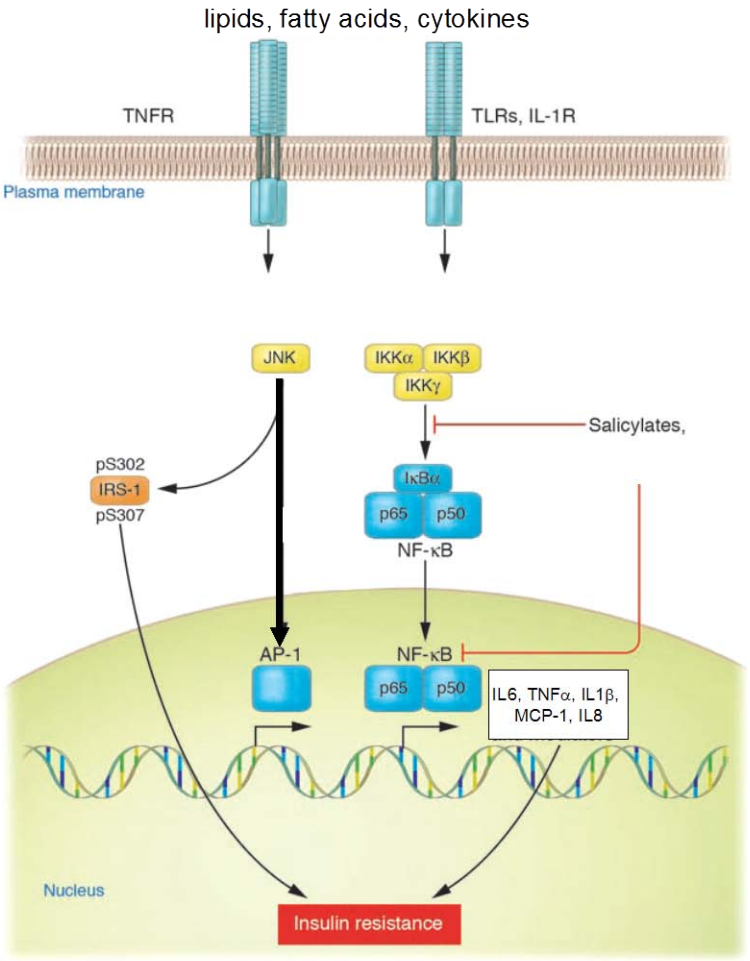
To assess the role of monocytes in insulin resistance we conducted a pilot investigation and utilized monocytes isolated from obese-IR, obese-IS, and nonobese-IS subjects for the purposes of assessing differences in expression of inflammatory genes in monocytes using RT-PCR.

Figure Legend

Figure 5.1 Possible stimuli for activating IKKb/NF-kB and JNK pathways in the obese state. Lipids, free fatty acids and cytokines can activate inflammatory pathways by binding to TNF-α, IL-1, or Toll receptors (TNFR, IL1R, or TLR). Degradation of the inhibitor of NF-κB (IκB) kinase β (IKKβ) allows NF-κB to the nucleus leading to increased expression of target genes that are potentially involved in the pathogenesis of IR. JNK also exerts its effects through inhibition of tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1). Adapted from "Inflammation and insulin resistance," by Shoelson S, et al, 2006, The Journal of Clinical Investigation, 116, p. 1794. Copyright 2006 by the American Society for Clinical Investigation.

Figure

5.1


lipids, fatty acids, cytokines
TNFR
TLRs, IL-1R
Plasma membrane
JNK
IKKα
IKKβ
IKKγ
Salicylates,
pS302
IRS-1
pS307
IκBα
p65
p50
NF-κB
AP-1
NF-κB
p65
p50
IL6, TNFα, IL1β, MCP-1, IL8
Nucleus
Insulin resistance

CHAPTER VI

6. Details of Research (Part 2)

6.1 Recruitment of adolescent and young adult subjects

Female adolescents and young adults between the ages of 16-25 years were recruited from the Pediatric Endocrine, Adolescent Medicine, and Nutrition clinics at the University of Massachusetts Medical Center in the fall of 2009. The subjects were interviewed by a trained interviewer and underwent a brief physical examination (Table 6.1). Persons with diabetes mellitus, any acute or chronic inflammatory disease as determined by clinical signs of infection, clinical evidence of either cardiovascular or peripheral artery disease, thyroid disease, Cushing´s disease or hypercortisolism, pregnancy, and those receiving medications that may affect our outcome parameters (metformin, lipid lowering medication, antihyperglycemic agents, steroids, non-steroidal anti-inflammatory drugs three days prior to blood draw) were excluded. Obese was defined as BMI > 95% for age. Nonobese was defined as BMI > 5% and < 75% for age.

A 40 mL sample of whole blood was obtained via venipuncture in the fasting state, and evaluated for metabolic and inflammatory parameters (Table 6.1). Insulin resistance was defined as homeostasis model of insulin resistance (HOMA-IR) ≥ 3.16. HOMA-IR is a surrogate measure of IR derived using the following formula: [Glucose (mg/dL) * Insulin (mIU)]/405. Several tools are available for defining IR, the gold standard being the hyperinsulinemic-

euglycemic clamp technique[52]. However, this method is invasive, complicated and time-consuming. The HOMA-IR is a more practical method to measure IR, and has been used in a large epidemiologic study in an adolescent population[53]. The cutoff of 3.16 was validated in a population of obese children and adolescents with IR as determined by an oral glucose tolerance test[54]. The study was approved by the University of Massachusetts Medical School Institutional Review Board.

6.2 Technical details

Blood sample collection and monocyte isolation: Blood samples were obtained via venipuncture after an overnight fast. Venous blood (40 mL total) was collected in six Vacutainer™ Cell Preparation Tubes (Becton Dickinson, Rutherford, NJ) with Na Citrate which are intended for the collection of whole blood and the separation of mononuclear cells using a FICOLL™ Hypaque™ solution. An additional 5 mL were collected in an EDTA tube (Vacutainer™, Becton Dickinson) for whole blood hematology analysis. Blood mononuclear cells were isolated from the Vacutainer™ Cell Preparation Tubes via centrifugation. Untouched primary monocytes were further isolated from the mononuclear fraction using a negative selection kit (Miltenyi Biotec, Germany).

RNA isolation: RNA was isolated from monocytes according to the QIAGEN MiniPrep protocol. cDNA from total RNA was synthesized using the iScript cDNA

Synthesis Kit (Bio-Rad Laboratories). Real-time PCR to quantify expression of inflammatory cytokines was performed using SybrGreen assays according to the manufacturer's instructions (Bio-Rad Laboratories). Expression of specific mRNAs was quantified in duplicate samples on an iCycler IQ Real-Time PCR detection system (Bio-Rad Laboratories) using the $\Delta\Delta$CT method with normalization to cycle threshold measurements for beta 2 microglobulin.

Serum cytokine quantification: Serum levels of leptin and inflammatory cytokines of interest (IL6, IL8, TNFα) were measured in fasting blood samples using the Milliplex™ immunoassay kit.

Statistical analysis: Baseline differences between obese-IS and obese-IR subjects were evaluated using the Student's *t* test for continuous variables and Fisher's exact test for categorical variables. In a secondary analysis, baseline differences between all obese (combined obese-IR and obese-IS) and non-obese-IS subjects were evaluated using the same methods. Variables with a skewed distribution (total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, fasting glucose, fasting insulin, HOMA, AST, ALT, CRP, total monocyte count, leptin) were log-transformed before analysis.

Tables

Table 6.1. Selected measures obtained at the study visit

Measure	Description
Demographcs	Age, sex, race,
Clinical data	Chronic medical conditions, current medications, family history of T2DM
Physical examination	Height, weight, blood pressure, waist circumference, pubertal staging
Fasting blood draw	Glucose, insulin, lipid panel, liver enzymes, complete blood count with differential, inflammatory markers, monocytes

CHAPTER VII

7. Experimental Evaluation (Part 2)

7.1 Study sample characteristics

Blood samples from eleven female subjects were used in this study. This pilot study was limited to females to minimize variability associated with gender. Using HOMA-IR analysis, we divided the subjects into three groups: obese-IR (n=4), obese-IS (n=3) and 4 nonobese-IS (n=4) (for definitions of IR and IS see 6.1 Adolescent Subject Recruitment"). The obese subjects were younger and displayed a larger waist circumference, higher systolic blood pressure, higher fasting insulin and more systemic leptin as compared to the nonobese-IS subjects (Table 7.1). The obese-IR subjects had, on average, a BMI that was higher than the obese-IS subjects, but this difference did not reach statistical significance (Table 7.1). Obese-IR subjects showed significantly elevated glucose, insulin and HOMA-IR as compared to obese-IS subjects (Table 7.1). All subjects provided written informed consent before taking part in the study.

7.2 Monocyte purity

Data were collected from the monocyte fraction and non-monocyte fraction of blood acquired from a healthy research volunteer. The purity of the enriched monocytes obtained using the Miltenyi^{TM} Monocyte Isolation Kit II was determined by flow cytometry. This kit uses an indirect magnetic labeling system to isolate all cells types except monocytes. Aliquots of the cell fractions were stained with fluorochrome-conjugated antibodies against a monocyte marker

(CD14-FITC), a B-cell marker (CD19-APC), and a T-cell marker (CD8-PE). The monocyte fraction contained 85% CD14+ cells (Figure 7.1), compared with the non-monocyte fraction which contained <1% CD14+ cells (Figure 7.2).

7.3 Monocyte proinflammatory phenotype

Expression of two cytokine signaling molecules (IL8, suppressor of cytokine signaling 3 (SOCS3)) and two downstream products of the JNK pathways (JunB, c-Fos) were measured from the CD14+ monocytes in our 3 subject groups. Both IL8 and SOCS3 showed a trend towards increased expression in the obese-IR subjects compared to the obese-IS and nonobese-IS subjects (Figure 7.3), suggesting the presence of a proinflammatory phenotype in monocytes in obesity, which is exacerbated in the insulin resistant state. Expression of JunB and c-Fos, two members of the Jun and Fos family of AP-1 transcription factors, trended higher in obese-IR patients than obese-IS and nonobese-IS subjects (Figure 7.4), indicating enhanced activation of the JNK pathway. In addition, the circulating levels of IL8, IL6 and TNFα were higher in obese-IR subjects compared to obese-IS subjects and nonobese-IS subjects (Figure 7.5).

7.4 Conclusions

The results from this pilot study in lean, obese-IS and obese-IR young females suggest that circulating monocytes are in a proinflammatory state in obesity, and this inflammation is exacerbated in the insulin resistant state. Our insulin

resistant cohort displayed an increased waist circumference, suggesting a larger visceral fat depot than the insulin sensitive patients. In addition, the elevated leptin level is not surprising as these patients likely have more fat mass than their peers in this study. The increased systemic levels of IL6 in the obese-IR cohort is consistent with cross-sectional data indicating a strong association between IR and IL6 levels[8].

The increased systemic level of IL8 as well as the increased expression of IL8 in monocytes of subjects in the obese-IR cohort suggest a potential role for IL8 in the pathophysiology of insulin resistance and a possible source of IL8 production. IL8, a member of the CXC chemokine family, is known to be stimulated by TNFα and secreted by adipocytes, particularly those in the visceral depot[55]. We have shown that in our sample of obese-IR young girls, elevated levels of TNFα may increase IL8 production and stimulation from circulating monocytes resulting in an increase in systemic IL8 levels which is consistent with other reports[56]. IL8 is thought to induce insulin resistance via the inhibition of insulin-induced Akt phosphorylation, providing a link between obesity and obesity-related comorbidities[55].

SOCS-3 inhibits the signal transduction of several cytokines, including leptin, via its disruption in the Janus-activating kinase2-signal transducer and activator of transcription 3 (JAK2-STAT3) pathway on leptin receptors[57]. Leptin resistance is

a common feature of obesity, and results in decreased fatty acid oxidation, excessive fat storage, and impaired satiety[58]. In addition to its role in leptin resistance, SOCS3 also inhibits insulin signaling by binding to phosphorylated Tyr^{960} of the insulin receptor[59], suggesting another potential role in the development of insulin resistance. Hepatic SOCS3 is known to be a mediator of insulin resistance in the liver[47,60] and our data suggest that SOCS3 may also play a role in altering monocyte insulin signaling. In addition, prior work in patients with chronic kidney disease reveals increased expression of SOCS3 in monocytes that is associated with increased plasma levels of IL6 and TNFα, and with progressive loss of renal function[61]. In this study, the authors speculate whether SOCS expression could be a marker of subacute cardiovascular disease, and a possible target for therapeutic intervention.

The JNK signaling pathway is known to be strongly associated with the development of obesity-associated inflammation[62]. JunB and c-Fos, two proteins with increased expression in our obese-insulin resistant cohort, can dimerize and form the AP-1 transcription factor and activate target genes, most notably those associated with inflammation[62]. This pathway is activated in adipose tissue and the liver in the obese state, and our data suggest that it is also activated in inflammatory cells, namely monocytes. This is consistent with prior work showing that inhibition of JNK in macrophages results in reversal of diet induced insulin resistance, without affecting obesity[44].

Our data are consistent with recent evidence suggesting that immune cells may be a key pathway whereby obesity and circulating free fatty acids contribute to systemic inflammation and insulin resistance. We showed that in female teenagers and young adults, obesity can induce a proinflammatory phenotype in circulating monocytes characterized by increased expression of proinflammatory cytokines, and proteins that are known to impair insulin sensitivity (Figure 7.6). In addition, these activated monocytes can be recruited to populate the adipose tissue as macrophages. Novel therapeutic interventions that decrease monocyte activation may delay or prevent obesity-related co-morbidities.

Table 7.1. Clinical and Biochemical Characteristics of Study Participants*

	Obese Insulin Resistant HOMA ≥ 3.16 (N= 4)	Obese Insulin Sensitive HOMA < 3.16 (N= 3)	Non-obese Insulin Sensitive HOMA < 3.16 (N= 4)	P Value Obese-IR vs. Obese-IS	P Value (Obese-IR + Obese-IS) vs. Non-obese-IS
Age, years	16.5 ± 0.5	17.7 ± 1.7	22.4 ± 3.3	0.22	**<0.01**
White race	25%	67%	50%	0.48	1
BMI§	42 ± 11	35 ± 6	22 ± 3	0.33	**0.01**
Waist circumference, cm	121 ± 24	103 ± 8	80 ± 8	0.26	**0.01**
Systolic blood pressure, mm Hg	130 ± 15	113 ± 4	104 ± 3	0.14	**0.03**
Diastolic blood pressure, mm Hg	73 ± 9	69 ± 15	66 ± 8	0.66	0.43
Medical History					
Polycystic ovarian syndrome	50%	33%	0%	1	0.24
First-degree relative with T2DM	75%	66%	25%	1	0.24
Fasting glucose, mg/dL	88 ± 8	70 ± 4	73 ± 8	**0.01**	0.32
Fasting insulin, mIU/mL	21 ± 7	5 ± 6	2 ± 1	**0.02**	**0.04**
HOMA-IR	4.6 ± 2	0.9 ± 1	0.45 ± 0.2	**0.02**	**0.04**
Total cholesterol, mg/dL	129 ± 33	121 ± 22	132 ± 42	0.81	0.79
LDL cholesterol, mg/dL	77 ± 19	64 ± 10	73 ± 35	0.4	0.9
HDL cholesterol, mg/dL	36 ± 8	44 ± 17	48 ± 7	0.61	0.21
Triglycerides, mg/dL	77 ± 42	69 ± 32	59 ± 17	0.93	0.7
AST, IU/L	18 ± 5	15 ± 3	16 ± 3	0.29	0.72
ALT, IU/L	24 ± 11	17 ± 7	14 ± 4	0.34	0.19
CRP, mg/L	5.3 ± 5	6.2 ± 8	<1 ± 0	0.88	0.17
Total monocyte count/mm3	658 ± 165	456 ± 105	375 ± 99	0.12	0.08
Leptin, pg/mL	4343 ± 3068	3409 ± 1850	1036 ± 702	0.89	**0.01**

* Plus-minus values are mean (± SD).

§ The body-mass-index (BMI) is the weight in kilograms divided by the square of the height in meters.

Figure Legend

Figure 7.1. Confirmation of monocyte purity. Flow cytometry showing the relative distribution profiles of CD14- (negative) and CD14+ (positive with anti-CD14 antibody conjugated to FITC) cells in the purified monocyte fraction. Data obtained from sample gated on forward and side scatter parameters that are appropriate for a monocyte population.

Figure 7.2. Confirmation of monocyte purity. Flow cytometry showing the relative distribution profiles of CD14- (negative) and CD14+ (positive with anti-CD14 antibody conjugated to FITC) cells in the purified monocyte-depleted (non-monocyte) fraction. Data obtained from sample gated on forward and side scatter parameters that are appropriate for a monocyte population.

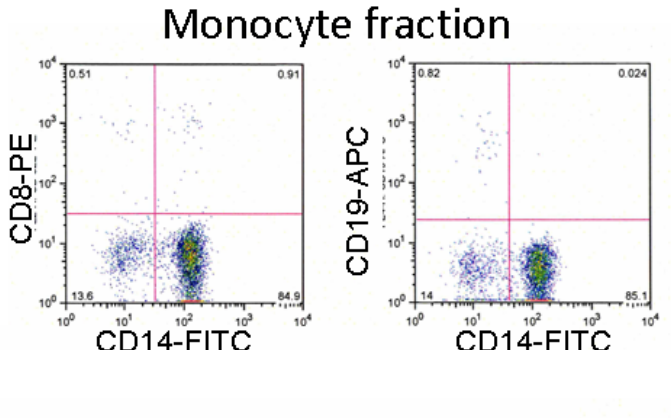
Figure 7.3. Expression of inflammation-related genes in the NFκB pathway in monocytes from non-obese (n=4), obese-IS (n=3) and obese-IR (n=4) subjects using qRT-PCR. Fold change in mRNA level of cytokines. Data were normalized to beta2-microglobulin. Error bars represent SEM.

Figure 7.4. Expression of inflammation-related genes in the JNK pathway in monocytes from non-obese (n=4), obese-IS (n=3) and obese-IR (n=4) subjects using qRT-PCR. Fold change in mRNA level of transcription factors. Data were normalized to beta2-microglobulin. Error bars represent SEM.

Figure 7.5. Serum levels of cytokines and chemokines in obese-IR, obese-IS and nonobese-IS subjects. Each data point represents an individual subject, the line represents the mean for that subject group. Data was unavailable for one patient in the obese-IS group due to technical difficulties.

Figures

7.1



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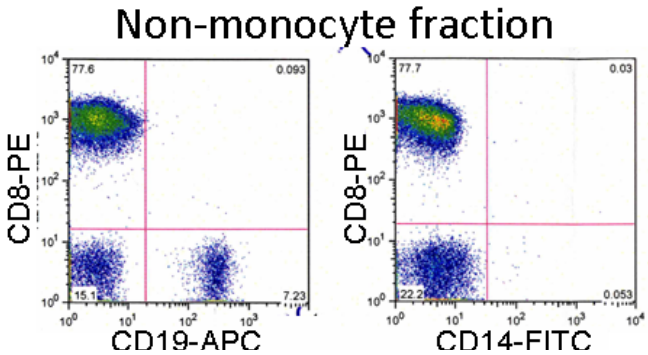

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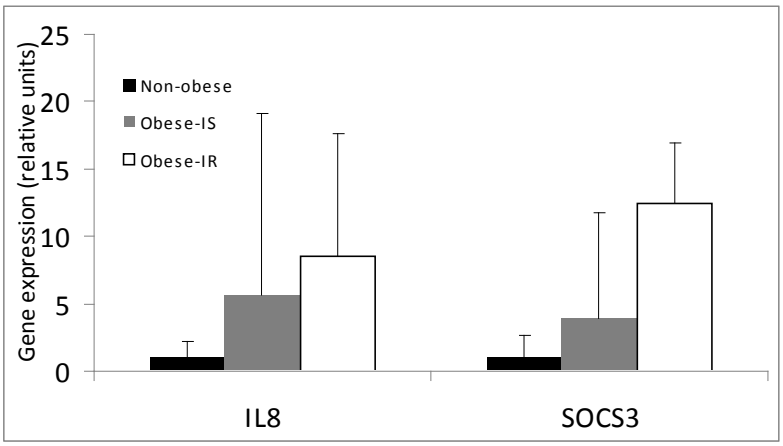
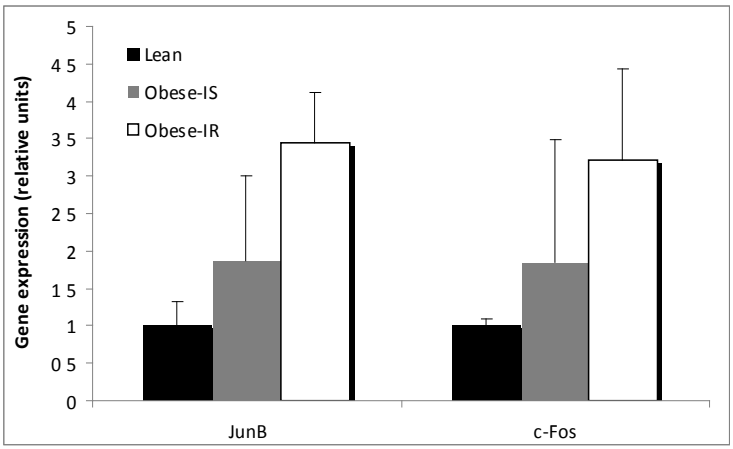


7.4



7.5

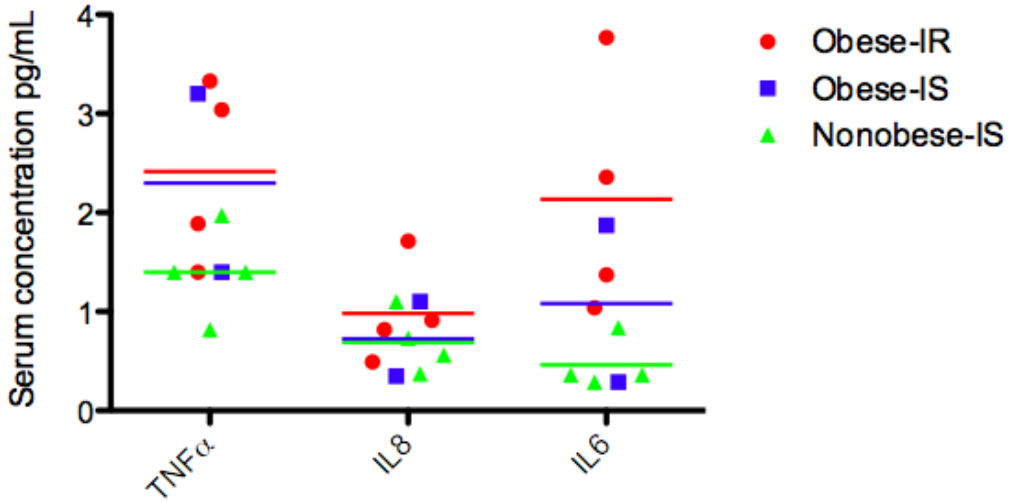
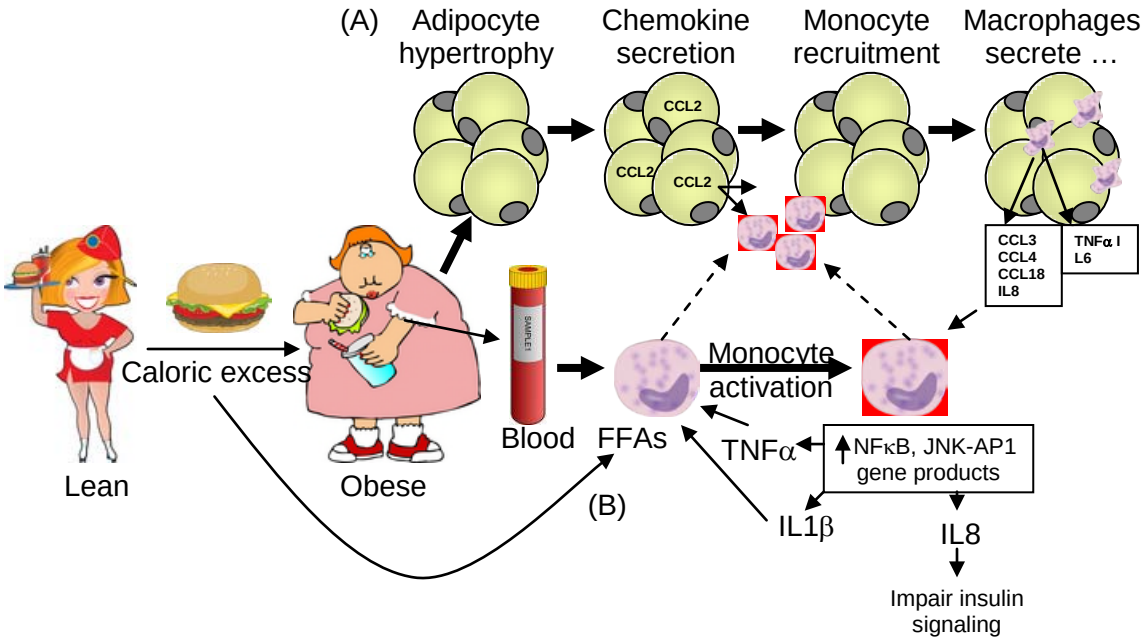


Figure Legend

Figure 7.6. Potential mechanisms linking obesity to immune cells and insulin resistance. (A) Caloric excess leads to adipocyte hypertrophy resulting in increased secretion of the monocyte chemoattractant CCL2. Monocytes are recruited to adipose tissue, differentiate into macrophages, and secrete chemokines that recruit additional monocytes, as well as cytokines that can activate monocytes, and impair insulin signaling. (B) Inflammatory pathways in circulating monocytes are activated by free fatty acids, TNFα or IL1β. Once activated, these monocytes can secrete cytokines that promote activation of other monocytes, or directly impair insulin signaling.

7.6


(A)
Adipocyte hypertrophy
Chemokine secretion
Monocyte recruitment
Macrophages secrete …
CCL2
CCL2
CCL2
CCL3
CCL4
CCL18
IL8
TNFα I
L6
Caloric excess
Lean
Obese
Blood
FFAs
Monocyte activation
TNFα
NFκB, JNK-AP1 gene products
IL1β
IL8
Impair insulin signaling
(B)

CHAPTER VIII

8. Final Summary and Future Work

This body of work implicates inflammatory cells, specifically macrophages and circulating monocytes, as key players in the pathophysiology of insulin resistance. The next step is to conduct a larger study to confirm the findings of monocyte inflammation observed in this pilot study in an adolescent population.

I am proposing to carry out a case-control study in young obese adolescent males and females between the ages of 12 and 18 years that will test the hypotheses that peripheral blood monocytes from obese-IR individuals (cases) when compared to obese-IS individuals (controls) demonstrate enhanced activation of inflammatory signaling pathways and proinflammatory cytokine secretion. The knowledge gained from this study will advance our understanding of the contribution of monocytes to the pathophysiology of IR in obesity, and may provide a readily measurable serum biomarker that can be used to identify high risk patients, track the progression of disease, and monitor responses to behavioral or pharmacologic therapies.

Specific Aim 1. Identification of a monocyte pro-inflammatory profile in obesity-induced IR. This aim will test the hypothesis that circulating monocytes from obese-IR individuals are more likely to exhibit a proinflammatory subtype as compared to monocytes from obese-IS individuals. We will use immuno-magnetic bead separation to isolate a population of pure monocytes from obese-

IR and obese-IS subjects. The distribution of pro-inflammatory $CD14^+CD16^+$ and classical $CD14^+CD16^-$ monocytes will be determined using flow cytometry to quantify the signature cell surface markers and chemokine receptors that distinguish these two monocyte subsets. These studies will characterize the distribution of two monocyte subtypes in the IR state.

Specific Aim 2. Critical evaluation of the capacity of monocytes from obese-IR individuals to secrete proinflammatory cytokines characteristic of obesity-induced IR. This aim will test the hypothesis that circulating monocytes from obese IR subjects have a stable potential for increased secretion and expression of pro-inflammatory cytokines as compared to monocytes from obese-IS individuals. Serum biomarkers of inflammation (TNFα, IL1β, IL6, IL8, MCP-1, IL10) will be measured in obese-IR and obese-IS youth. Spontaneous and stimulated secretion from cultured monocytes isolated from obese-IR subjects will be critically evaluated and compared to corresponding data from obese-IS subjects. Gene expression of key inflammatory cytokines will be measured in monocytes using quantitative PCR. These studies will establish a potential role for monocytes in the development of systemic inflammation seen in obese-IR.

Specific Aim 3. Identification of inflammatory signaling pathways that are activated in monocytes in obesity-induced IR. This aim will test the hypothesis that IKKβ-NFκB and JNK-AP1 signaling pathways are activated in monocytes from obese-IR patients as compared to monocytes from obese-IS individuals.

Immunohistochemistry will be used to assess localization of key transcription factors. In addition, phosphorylation states of specific proteins in these two pathways will be analyzed. These studies will provide insights into the molecular alterations present in monocytes from obese-IR individuals, and may identify potential therapeutic targets to ameliorate the inflammatory state.

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